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<http://dx.doi.org/10.1289/ehp.1408504>

Received: 1 April 2014

Accepted: 17 December 2014

Advance Publication: 19 December 2014

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National Institute of
Environmental Health Sciences

Developmental Exposure to a Commercial PBDE Mixture: Effects on Protein Networks in the Cerebellum and Hippocampus of Rats

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Running title: Proteomics and DE-71 developmental neurotoxicity

Acknowledgments: The authors thank Dr. June Dunnick of NIEHS, Research Triangle Park, NC, Dr. Carol Parker from UNC Proteomics Center, Chapel Hill, NC and Dr. Henrik Viberg of Uppsala University, Uppsala, Sweden for their comments on an earlier version of this manuscript. The proteomics work was conducted under EPA contract EP06D000284. The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Preliminary findings were presented at the 27th International Symposium on Halogenated Environmental Organic Pollutants and POPs (Dioxin-2007), Tokyo, Japan (Sept 3-7, 2007).

Competing financial interests: The authors declare they have no actual or potential competing financial interests.

Abstract

Background: Polybrominated diphenyl ethers (PBDEs) are structurally similar to polychlorinated biphenyls (PCBs) and have both central (learning and memory deficits) and peripheral (motor dysfunction) neurotoxic effects at concentrations/doses similar to those of PCBs. The cellular and molecular mechanisms for these neurotoxic effects are not fully understood; however, several studies have shown that PBDEs affect thyroid hormones, cause oxidative stress, and disrupt Ca^{2+} -mediated signal transduction. Changes in these signal transduction pathways can lead to differential gene regulation with subsequent changes in protein expression, which can affect the development and function of the nervous system.

Objective: In this study, we examined the protein expression profiles in the rat cerebellum and hippocampus following developmental exposure to a commercial PBDE mixture, DE-71.

Methods: Pregnant rats (Long Evans) were dosed perinatally with 0 or 30.6 mg/kg/day of DE-71 from gestation day 6 through sampling on postnatal day (PND) 14. Proteins from the cerebellum and hippocampus were extracted, expression differences were detected by two-dimensional difference gel electrophoresis (2D DIGE), and proteins were identified by tandem mass spectrometry (MS/MS). Protein network interaction analysis was performed with Ingenuity[®] Pathway Analysis and proteins of interest were validated via Western blotting.

Results: Four proteins were found to be significantly differentially expressed in the cerebellum following DE-71 exposure while 70 proteins were significantly differentially expressed in the hippocampus. The identified differentially expressed proteins (four from the cerebellum and 47 from the hippocampus) had roles in mitochondrial energy metabolism, oxidative stress, apoptosis, calcium signaling, and growth of the nervous system.

Conclusions: Results suggest that changes in energy metabolism and processes related to neuroplasticity and growth may be involved in the developmental neurotoxicity of PBDEs.

Introduction

Polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in domestic and industrial applications including computers, television sets, and mobile phones; also in furniture, textiles, insulation boards, mattresses, and upholstery furnishings (Alaee et al. 2003). Like polychlorinated biphenyls (PCBs), PBDEs are structurally similar synthetic chemicals comprised of two phenyl rings linked by oxygen (thus the designation as “ethers”; see Supplemental Material, Figure S1). They are ubiquitous in the environment, where they bioaccumulate, becoming toxic to animals and humans (Kodavanti et al. 2008). Levels of PBDEs have been reported to be increasing in some parts of the environment, in human blood, and milk (McDonald 2005).

PBDEs are typically produced for industrial use at three different levels of bromination, *i.e.*, penta-, octa- and deca-brominated diphenyl ether mixtures (La Guardia et al. 2006; WHO 1994). Commercially available PBDE products are not single compounds or even single congeners but rather a mixture of congeners. The commercial PBDE mixture, DE-71, consists of over 20 different congeners. The primary constituents include 2,2',4,4'-tetrabromodiphenyl ether (PBDE 47, ~38%) and 2,2',4,4',5-pentabromodiphenyl ether (PBDE 99, ~49%). Collectively these two congeners account for ~87% (w/w) of the DE-71 mixture (La Guardia et al. 2006). In the U. S. A., PBDE 47 and PBDE 99 are the 2 most predominant congeners detected in human milk, serum and whole blood (Schechter et al. 2005). Like other lipophilic compounds, PBDEs readily cross the placenta into the fetus and through milk during lactation, providing an opportunity for PBDEs to interfere with developmental processes (Kodavanti et al. 2010; Mazdai et al. 2003).

Several studies have shown that PBDE exposure results in alterations in spontaneous behavior and reduced learning and memory in mice (Viberg et al. 2003a; Viberg et al. 2003b; Viberg et al. 2004). These effects were similar to those seen after neonatal exposure to the structurally related chemicals, the PCBs (Eriksson and Fredriksson 1996). Rice and collaborators (Rice et al. 2007) have shown developmental delays in the acquisition of the palpebral reflex following repeated neonatal exposure to PBDE 209, along with changes in circulating thyroxine (T4) levels. However, mice exposed to PBDE 47 on postnatal day (PND) 10 display a delayed ontogeny of neuromotor functional endpoints plus adult hyperactivity (Gee and Moser 2008). Considering critical neurodevelopment effects including habituation response for PBDEs, the derived RfD values were 0.1, 0.1, and 0.2 $\mu\text{g}/\text{kg}/\text{day}$, respectively for PBDEs 47, 99, and 153 (IRIS 2008).

In a previous study on the same cohort of animals (Kodavanti et al. 2010), we found that DE-71 was associated with a significant decrease in circulating T4 levels and accumulation of PBDE congeners in various tissues, including the brain. This suggests that PBDEs cross the blood-brain barrier, potentially causing changes in neurobehavioral parameters (Kodavanti et al. 2010). In addition, we have reported that PBDEs, like PCBs, affect intracellular signaling pathways including calcium homeostasis, mitogen-activated protein kinase, and translocation of protein kinase C (PKC) (Fan et al. 2010; Kodavanti et al. 2005). All of these signaling pathways are known to be associated with the development of the nervous system and to be involved in learning and memory (Kater and Mills 1991; Lein et al. 2007). Perturbations of such signal transduction pathways in the brain could affect gene regulation and alter protein expression, which might have an ultimate effect on nervous system growth and function. In this study, the protein expression profiles in the cerebellum and hippocampus of rats exposed to DE-71

perinatally were examined using 2D difference gel electrophoresis (2D DIGE)-based quantitative intact proteomics (QIP).

Materials and Methods

Animals and chemical exposure

Timed pregnant Long-Evans Hooded rats (approx. 230 g; Charles River Labs, Portage, OR) were obtained on gestational day 3 (GD 3; day of insemination = GD 0) and were housed individually in standard polycarbonate plastic cages with heat-treated pine shavings as bedding under regulated temperature ($21 \pm 2^\circ\text{C}$), relative humidity ($50 \pm 10\%$) and 12 h light/dark cycle. Food (Purina lab chow #5008 lactation; #5001 post-weaning) and water were provided *ad libitum*. (Kodavanti et al. 2010). The protocols and the use of animals in all experiments were approved by the animal care and use committee of the National Health and Environmental Effects Research Laboratory at the U.S. Environmental Protection Agency, and the animals were treated humanely and with regard for alleviation of suffering.

The commercial PBDE mixture DE-71 (Lot # 1550OI18A) was a gift from Great Lakes Chemical Corporation (El Dorado, AR). The presence of impurities, including brominated biphenyls, dioxins and furans, has been reported elsewhere (Hanari et al. 2006). The dose of DE-71 (30.6 mg/kg/day in corn oil) was selected to match, on a molar basis, the doses of Aroclor-1254 from previous studies for which we have extensive information, both *in vitro* and *in vivo* (see review Kodavanti 2005). This allows us to compare the effects between these two structurally related groups of chemicals. Dams (N = 15/dose group) were weighed and given DE-71 in corn oil or corn oil alone (2 ml/kg body weight) by oral gavage daily (between 8:00 and 10:00 AM) from GD 6 through PND 14, except on PND 0 when the dams were not disturbed.

Dams delivering a litter of 10-15 pups were used in the study. On PND4, litters were culled to 8 pups with a minimum of five males.

The reproductive outcomes, decreases in circulating T4 levels, assignment of rats for different measures, general health, and development of the rats used in these studies as well as biological relevance of the highest dose used in this study in terms of human levels have been previously reported (Kodavanti et al. 2010). No changes in maternal or pup body weights or pup motor activity were found. For the present studies, one male pup each from three different litters at PND 14 were sacrificed by guillotine, cerebella and hippocampi were dissected, quickly frozen on dry ice, and stored at -80°C until further processing. Dissections were carried out in a necropsy room at 10 am in the animal facility following morning dosing and weighing protocols.

Protein extraction

Preparation of samples for proteomic analysis was as previously described (Kodavanti et al. 2011). Briefly, each tissue fraction (approximately 40 mg) was suspended in 200 μL of lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris pH 7.5, supplemented with protease inhibitor Complete[®] (Pierce, Rockford, IL) and phosphatase inhibitor, NaVO_4 (Sigma, St. Louis, MO)). Preparation of protein lysates was performed with a 2D-clean up kit (GE Healthcare, Piscataway, NJ) and the resulting pellet was resuspended in focusing buffer (8M urea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5). Protein concentration was determined with GE Healthcare's 2D-Quant kit as indicated by the manufacturer.

2D DIGE analysis

Differential protein expression was determined with 2D DIGE-based QIP (Alban et al. 2003; Friedman et al. 2004) utilizing a 3 dye protocol that allows simultaneous labeling of an internal

control, treated, and untreated brain samples. This protocol has been shown to greatly reduce the variability inherent in 2D gel protein analysis. All experimental procedures were as previously described (Kodavanti et al. 2011). A total of three control and three treated brains were used. Briefly, 120 μ g of total protein from each treated sample and from each control sample were labeled with 400 pmol of CyDyes Cy-5 and Cy-3 (GE Healthcare), respectively. The internal control was created by pooling 60 μ g from control and treated samples followed by labeling with 400 pmol of Cy-2.

Isoelectric protein separation of labeled samples ($n = 3$ animals/group) was performed using immobilized pH gradient (IPG) strips (13cm, pI range 3-10; GE Healthcare). Protein separation in the second dimension was done with SDS (12%)-PAGE gels (4% stacking) casted on low fluorescence glass plates (13 cm, GE Healthcare). Each control sample was run against each experimental sample for a total of 9 gels, improving statistical power. The resulting gels were scanned using a Typhoon 9410 imaging system (GE Healthcare) and then were fixed and stained with colloidal Coomassie for further processing.

Protein identification

Protein identification was performed as previously described (Winnik et al. 2012). Briefly, spots with statistically significant expression changes as determined by the DeCyder 2D software (GE Healthcare) (see Statistics section below) were excised from the gels with the Ettan Spot Picker (GE Healthcare). In-gel protein digestion was carried out using modified trypsin, and MALDI MS/MS data were acquired either with a 4700 or a 4800 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems). The proteins were identified by a combination of peptide mass fingerprinting and the sequence-tag approach (DeKroon et al. 2011). The peptide mass fingerprinting and sequence tag data were evaluated either with Mascot scores (Matrix

Science 2013) or the online proteomic software "Aldente" (Winnik et al. 2012). Keratin and trypsin autolysis contaminant peaks were identified and excluded from the MS/MS analysis. This step was followed by MALDI MS/MS sequencing, and the final protein identification was done using Protein Pilot 3.0 software (Applied Biosystems) and the Mascot[®] Search Engine (Matrix Science), searching against the rat species database within the non-redundant database (NCBIInr) or the SwissProt database (ExPasy Bioinformatics Resource Portal 2013). Finally, mass-selected MALDI MS/MS spectra acquisition was performed to further increase protein sequence coverage. Proteins were identified based on at least two unique MS/MS peptide sequences with confidence scores larger than 95% (corresponding to the overall minimum protein identification confidence level of 99.75%) based on Protein Pilot 3.0 peptide and protein % confidence score criteria. Alternatively, a MS/MS-based MASCOT protein score of 40 or higher was considered to be acceptable (Winnik et al. 2012).

Western blot analysis

Each sample was prepared as described previously (Kodavanti et al. 2011). In brief, 200 mg of tissue was sonicated in 200 μ l RIPA buffer (Sigma) with Complete[®] protease inhibitor cocktail from Roche's and spun at approximately 20,000 x g for 10 min at 5 °C. The supernatant was collected and protein content was determined with a 2D Quant kit (GE Healthcare). A 10- μ g aliquot of total protein was separated on 10% SDS-PAGE gels, the proteins were transferred to PVDF membranes for 16 hr in a cold room, and the membranes were probed with monoclonal antibodies from Abcam, (Cambridge, MA) against anti-Protein disulfide isomerase A3 (PDIA3) (ab10287) and α -tubulin (ab7291) as an internal loading control (Alban et al. 2003). The protein PDIA3 was chosen for analysis by Western blot to validate the 2D gel data set because it

possesses multiple functional roles in CNS physiology that were impacted by gestational and lactational exposure to DE-71.

Protein functional analysis

NCBI data bases ([National Center for Biotechnology Information](#), [U.S. National Library of Medicine](#), Bethesda MD) were used to research protein functional ontology. This information was used to separate the differentially expressed proteins into functional groups. Follow-up analysis with Ingenuity Pathway Analysis software v9.0-3210 (Ingenuity) provided additional correlations between identified proteins and was used to generate functional networks as described previously (Kodavanti et al. 2011). Networks are ranked by their scores which are based on a p -value calculation on the probability that network proteins are part of the network by random chance and are equal to the negative exponent of this calculation. For example, a score of 25 reflects a highly relevant networks with p -values of 10^{-25} . Canonical overlays had p -values ≤ 0.05 .

Statistics

The digital images from the Typhoon system were analyzed with DeCyder 2D software (GE Healthcare) to determine changes in protein levels across gels and for pair-wise comparisons of individual Cy3- and Cy5-labeled samples (Friedman et al. 2004). Pair-wise comparisons of each PND 14 control and the corresponding DE-71 sample with the pooled internal standard present on each gel was performed with the DeCyder differential in-gel analysis (DIA) module. Two standard deviations from the mean volume ratios (95th percentile confidence) were used as a threshold to determine the levels of significance for a given set of samples. Statistical analysis and gel-to-gel comparisons were performed with Decyder's Biological Variation Analysis (BVA)

module (DeKroon et al. 2011). Data were log2 transformed and protein changes between control and DE-71 treated rats were analyzed using Student's t-test ($p < 0.05$). Fold changes calculated as the ratio of the treated to the control were derived on the mean expression changes determined. Significance for Western analysis was determined by t-test at $p \leq 0.05$ (Microsoft® Excel® 2013).

Results

Differential protein expression analysis

The perinatal DE-71 exposure had a significant impact on cerebellar and hippocampal protein expression at PND14. Representative examples of 3 dye-stained gels as scanned by the Typhoon 9410 imaging system (GE Healthcare) with significantly different spots identified for the cerebellum and hippocampus are presented in Figure 1. These gel images, analyzed by DeCyder 2D software (GE Healthcare), resulted in 3D images representing changes in protein levels in the cerebellum and hippocampus (Figure 2A and B, respectively). Analysis of the 3D images identified 4 and 70 protein spots as differentially expressed in the cerebellum and hippocampus, respectively.

Proteins available in amounts sufficient for downstream procedures were identified by MALDI TOF/TOF mass spectrometry analysis. This included 4 of the 4 cerebellar protein spots and 47 of the 70 differentially expressed hippocampal protein spots ($p < 0.05$) (Figure 1). Protein identification features including accession number, isoelectric point, molecular weight and Mascot protein score, are presented in Supplemental Material, Table S1. Seven of the identified hippocampal proteins had two post-translational modifications (Albumin [ALBU], Glial fibrillary acidic protein [GFAP], Dihydropyrimidinase-related protein [DPYL] 2 & 5, Heat shock

protein [HSP] 74, Creatinine kinase B-type [KCRB], and Tubulin alpha1-B [TBA1B]), one had two isoforms (Glyceraldehyde-3-phosphate dehydrogenase [G3P]), and one protein (DPYL3) was identified in 4 isoforms, thus the final count for identified hippocampal proteins is 35. All of the cerebellar proteins (4/4) (see Supplemental Material, Table S2) and ~ 94% (66/70) of hippocampal proteins (see Supplemental Material, Table S3) affected, were up regulated by DE-71 exposure.

Protein functional analysis

Utilizing the NCBI and Swiss-Prot data bases, individual protein functions were ascertained based on the specific accession numbers determined from protein identification software (DeCyder 2D software, GE Healthcare). Proteins then were grouped into larger functional categories by correlating changes in expression with possible functional response to and consequences of DE-71 exposure. Differentially expressed proteins in the cerebellum were related to growth functions associated with carbohydrate metabolism (Alpha-enolase [ENOA] and Fructose-bisphosphate aldolase C [ALDOC]) and RNA processing (Heterogeneous nuclear ribonucleoprotein [HNR] H2 and Heterogeneous nuclear ribonucleoprotein A3 [ROA3]) (see Supplemental Material, Table S2). Growth functions were also prominently displayed in the identified hippocampal proteins with 18 of the 35 identified proteins falling into this general category. Proteins with roles in cytoskeleton assembly (e.g. Septin-5 [SEPT5], myristoylated alanine-rich-C-kinase [MARCKS]), energy metabolism (e.g. KCRB), neurotransmission (e.g. Glutamine synthetase [GLNA]) and cell division (Transitional endoplasmic reticulum ATPase [TERA]), as well as proteins with roles in common with those identified in the cerebellum such as carbohydrate metabolism (ENOA and gamma-enolase [ENOG]) and RNA processing (HNRH1) are included in this group. The next major functional group in the hippocampus

related to proteins linked to the stress response and is indicative of the adverse effects of DE-71 exposure. Proteins markers of neuronal death (GFAP) and microglial cell activation (ALBU); heat shock proteins that act as protein chaperones (e.g. HSP74 and HSP7C); and Protein disulfide-isomerase A3 [PDIA3], a protein involved in redox metabolism were differentially expressed in DE-71 exposed rat hippocampi. Several proteins with roles in neuronal plasticity, possibly in response to damage, were also identified. For example, proteins involved in neuronal development (e.g. DPYL2, 3 & 4 or Dynactin subunit 2 [DCTN2]) and synaptogenesis (DCTN2) were up-regulated. The final functional group relates to protein chemistry and is comprised of proteins with roles in protein catabolism (Proteosome subunit alpha type-1 [PSA1]), complex assembly (Glucose regulated protein 78 kDa [GRP78]), formation of secondary structure (T-complex protein 1 subunit [TCP] epsilon and gamma) and synthesis (Eukaryotic translation initiation factor 4B [Q5RKG9]).

The results of the Ingenuity pathway analysis, carried out on the list of identified hippocampal proteins, agreed closely with the functional ontology results. The network in Figure 3A represents the top network with a score of 25 ($p < 10^{-25}$). Fourteen of the 35 identified proteins fell within this network which included proteins involved in “post-translation modification, protein folding and drug metabolism”. Canonical overlays highlighted the nuclear factor erythroid 2-related factor (Nrf2)-mediated oxidative stress, glucose metabolism and protein modification pathways. The network in Figure 3B shows the results from merging three of the remaining top networks with scores of 17, 9 and 2 ($p < 10^{-17}$, 10^{-9} , and 10^{-2} , respectively), which included proteins with roles in cellular function and maintenance, cellular assembly and organization, and nervous system development and function. The remaining DE-71 affected hippocampal proteins are found in this network. For example, the enolases ENOA (ENO1) and G

(ENO2) participate in glucose metabolism and EIF4B, which binds ribosomes and functions in synthetic processes, contribute to cell growth and maintenance. Tubulin and the Dihydropyrimidinase-related proteins (DPYSL2, 3 and 5) have roles in cytoskeleton and axonal growth contribute to both cell organization and neuronal development. Similar functions can be identified for the other proteins (see Supplemental Material, Table S3). Canonical and functional overlays include protein modification and glucose metabolism as in network I, but also include signaling and neuronal functions.

Western blot confirmation

Supplemental Material, Figure S2A and S2B shows the validation of 2D-DIGE results with Western blot analysis of PDIA3 (also called as ERP57) in control (0 mg/kg) and DE-71 dosed (30.6 mg/kg) rat hippocampus. Panel A displays the mean fold-change (1.78 ± 0.28 (SE)) in the hippocampus by DE-71 exposure versus controls from three independent experiments ($n = 3$). These data closely match that of the 2D gel study (1.67-fold increase, see Supplemental Material, Table S3). Panel B shows representative protein bands from control and treated hippocampus for PDIA and the housekeeping protein, α -tubulin, used as an internal control.

Discussion

There is growing evidence that PBDEs have an adverse impact on neurobehavioral and functional development. Epidemiological studies have shown that prenatal PBDE exposure was associated with reduced development in children, including both psychomotor development and full scale IQ performance (Eskenazi et al. 2013; Herbstman et al. 2010). These epidemiological studies are supported by animal studies where PBDE exposure delayed the ontogeny of neuromotor function, resulting in hyperactivity in adult mice (Gee and Moser 2008). Viberg et

al. (2003a; 2003b; 2004) reported persistent aberrations in spontaneous behavior and habituation capability in mice after a single developmental exposure (PND 10) with PBDEs 99, 153, and 209. Studies in our laboratory on DE-71 developmental exposure found accumulation of PBDE congeners in various tissues including brain, were associated with subtle changes in parameters of neurobehavior, decreased circulating T4 levels (Kodavanti et al. 2010). Perturbed thyroid hormone homeostasis, altered cell signaling including calcium homeostasis, and neurotransmitter changes have been postulated as critical events leading to the adverse neuronal effects of persistent organic pollutants such as PBDEs (Kodavanti 2005). In this study, we have examined changes in protein profiles in the cerebellum and hippocampus following DE-71 exposure. The results indicated that perinatal exposure to DE-71 altered the expression of proteins associated with key metabolic pathways involved in cell growth, protein metabolism, neuronal plasticity, and stress responses (see Supplemental Material, Tables S2 and S3; Figure 3). The effect on the hippocampus as measured by number of affected proteins was greater than on the cerebellum, most likely due to ontogenetic differences in the two brain areas during time of exposure (GD6 to PND14). In contrast to most of the brain regions, both the hippocampus and cerebellum develop largely postnatally (Buell et al. 1977). However, postnatal hippocampal neurogenesis continues at some level for up to 3 months of age in the rat, while cerebellar neurogenesis begins to decline at about 6 days post birth (Altman and Das 1965).

Maintaining normal cell function requires an adequate energy supply, especially during growth and under conditions of stress. Differential expression of multiple proteins with roles in energy production was found after DE 71 exposure. Glycolysis seemed to be mainly affected, suggesting greater changes in the levels of cytoplasmic proteins. ENOA, a component of glycolysis and generates ATP through high energy intermediates (Wold 1971), was significantly

upregulated both in the cerebellum and hippocampus. A similar increase has been reported by Alm et al. (2006) in the mouse hippocampus following a single oral dose of PBDE 99 at postnatal day 10. In the same pathway, ALDOC was upregulated in the cerebellum while G3P and phosphoglycerate kinase 1 (PGK1) were upregulated in the hippocampus. ALDOC splits fructose 1,6-bisphosphate into the dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. G3P and PGK1 catalyze the sixth and seventh steps of glycolysis, generating ATP. G3P is also the precursor to pyruvate which feeds into the Krebs cycle. This is particularly important in the brain that uses primarily mitochondrial oxidative phosphorylation as its source of ATP. Pathway analysis identified gluconeogenesis/glycolysis to be one of the highest ranked altered pathways in this data set (Figure 3). Additional proteins related to glycolysis (pyruvate dehydrogenase E1, ODPB), the Krebs cycle (malate dehydrogenase, MDHC), electron transport (NADH-ubiquinone oxidoreductase, NDUS1) and energy transduction (KCRB) were also differentially expressed in the hippocampus. KCRB is specific for brain tissue and plays a major role in generating ATP under conditions of high energy demand (Mahadevan et al. 1984). Although creatine kinase is widely regarded as a soluble enzyme, the brain isoform is associated with synaptic plasma membrane (Lim et al. 1983). It has been shown to be regulated by thyroid hormones, with increased serum KRCB levels being associated with hypothyroidism (Beyer et al. 1998). Developmental DE-71 exposure is known to cause hypothyroidism by decreasing circulating T4 levels (Kodavanti et al. 2010) and this effect could play a role in the alterations of KRCB seen in the hippocampus. Mukherjee et al. (2013) recently reported that PBDE-47 altered several proteins related to energy metabolism including ENOA. PBDE-154 has been shown to deplete mitochondrial ATP by interacting with the inner mitochondrial membrane, inhibiting electron transport and reducing the membrane potential (Pereira et al. 2014) and PBDE-49 inhibited

electron transport complexes IV and V in brain mitochondria (Napoli et al. 2013). These data highlight the critical role of energy metabolism in DE-71's mode of action.

In addition to ENOA, proteins related to nucleotide metabolism were also altered both in the cerebellum and hippocampus. HNRH2 and HNRH1 were up-regulated in the cerebellum and hippocampus, respectively. In addition, ROA3 was increased in the cerebellum while heterogeneous nuclear ribonucleoprotein K (HNRHK) was increased in the hippocampus by DE-71. These proteins have multiple functions in the processing of heterogeneous nuclear RNAs into mature mRNAs and can act as trans-factors in regulating gene expression (Chaudhury et al. 2010).

In another growth-related function, several hippocampal proteins related to cytoskeleton structure and axonogenesis were changed by DE-71 (see Supplemental Material, Table S3). Tubulin alpha-1B chain (TBA1B), tubulin alpha-1C (TBA1C), SEPT5 and neurofilament light polypeptide (NFL) were upregulated, while MARCKS was down-regulated. Along with actins, tubulins are abundant cytoskeletal proteins that support diverse cellular processes including microtubule and microfilament structure and function (Lundin et al. 2010). Septins are GTP-binding proteins with roles in vesicle trafficking, apoptosis, remodeling of the cytoskeleton, neurodegeneration, and neoplasia (Hall et al. 2005). MARCKS protein has been implicated in actin cytoskeletal rearrangement in response to extracellular stimuli (Eun et al. 2006). Mukherjee et al. (2013) reported a similar increase in cytoskeletal proteins (tubulin beta chain and actin) following BDE-47 exposure in the snail, *Crepidula onyx*. In neural stem/progenitor cells, BDE-209 and/or BDE-47 decreased the expression of cytoskeletal proteins such as cofilin-1 and vimentin (Song et al. 2013). Together, these data suggest perturbed neuronal processes, either as a disruption of normal neurite outgrowth or possibly as a repair response to the DE-71-induced

neurotoxic effects, in agreement with Viberg and Eriksson (2011) where changes in proteins involved in maturation of brain, neuronal growth and synaptogenesis were associated with changes in learning and memory by PBDE exposure.

Further evidence of an impact on neurogenesis can be seen with the effect of DE-71 on the dihydropyrimidinase-related proteins (DPYL2, DPYL3 and DPYL5) for which multiple isoforms were affected. These proteins are involved in facilitating neuronal growth cone migration, promoting microtubule assembly, and have a role in synaptic signaling. They are required for signaling by the cell-adhesion semaphorin proteins (Suzuki et al. 2003). Dihydropyrimidinase-related proteins have been targets for oxidative stress in Alzheimer's disease brains (Castegna et al. 2002) and both DPYL proteins and their companion semaphorins have been shown to be up-regulated in response to nervous system injury (Murphey et al. 1999). Ingenuity pathway analysis identified axonal guidance, semaphorin signaling, and neurodegenerative process as important pathways in DE-71 neurotoxicity (Figure 3). Also involved in synaptic plasticity, the dynactin protein, DCTN2, was upregulated by DE-71. Dynactin is necessary for stabilization of the synapse and for dendritic arborization during development (Eaton et al. 2002; Murphey et al. 1999). Dingemans et al. (2007) showed that PBDE-47 exposure reduced long-term potentiation together with changes in postsynaptic proteins involved in synaptic plasticity in the mouse hippocampus.

Multiple proteins up-regulated in this study suggest possible pathways for PBDE-induced neurotoxicity. For example, GFAP, a marker of neuronal damage, was up-regulated. GFAP is expressed in the central nervous system in astrocytes and is involved in cell communication and the functioning of the blood brain barrier. However, following neuronal damage, astrocytes infiltrate the damaged area and GFAP levels increase (O'Callaghan and Sriram 2005). Increases

in brain GFAP have been documented following exposure to environmental chemicals (Brock and O'Callaghan 1987) and have become an accepted biomarker for neurotoxicity (O'Callaghan and Sriram 2005).

Protein metabolism was heavily influenced by DE-71 exposure. Our data show upregulation of proteins involved in protein folding (TCPG) and complex assembly (GRP78). In addition, a number of proteins important in the brain's response to stress were affected. In the hippocampus, proteins related to the chaperone/ubiquination pathway were increased by DE-71 (see Supplemental Material, Table S3). Heat shock proteins (HSPs) such as HSP74, HSP7C, HSP105 were all increased approximately 1.6 to 1.7 fold in DE-71-treated animals relative to untreated controls (60-70% increase over controls). HSPs are molecular chaperones with roles in protein synthesis, protect against stress by aiding in refolding of slightly damaged proteins, or can transfer severely damaged proteins to the proteasome for degradation (Becker and Craig 1994). A number of ontology groupings by Ingenuity highlighted protein ubiquination pathways as significantly impacted in the hippocampus by DE-71 (Figure 3).

Of particular interest, the only two proteins with decreased expression following DE-71 exposure have roles in immune cell activation. As described above, MARCKS has a role in cytoskeleton rearrangement in response to external stimuli. Induction of MARCKS also has been observed following microglial activation, linking it to neurodegenerative processes (Murphy et al. 2003). However, current results indicate a decrease (-1.39 fold) in MARCKS protein. The second down-regulated protein, PSA1 (also known as Macropain, -1.40 fold), in addition to its role in protein degradation as a component of the proteasome, is known to mediate the LPS-induced activation of macrophages with consequent transcription of genes that encode pro-inflammatory regulators

of the immune response (Martinez-Solano et al. 2009). Thus, depletion of these proteins by DE-71 could negatively impact the brain's capacity to mount a defense against damage and/or insult.

It is important to note that there is considerable cross over in secondary functions for many of the proteins listed. For example, the enolases were grouped in Supplemental Material, Table S3 under “cell growth and function” because of their roles in carbohydrate metabolism and glycolysis, but also serve roles in stress response in neurons (Diaz-Ramos et al. 2012). Glutamine synthetase is important in the brain for GABA synthesis, and also is a significant player in energy metabolism as part of the citric acid cycle. Proteins with general roles in cell proliferation, in the nervous system often have roles in neuronal function. For example, DCTN2 is important in chromosome alignment during mitosis (Quintyne et al. 1999), and may also play a role in neurogenesis and synaptogenesis during brain development or plasticity following damage. Dynactin is important in linking Sun1/2 and Syne/Nesprin-1/2 to the nuclear membrane, proteins important to the nuclear movement that is critical for neurogenesis and neuronal migration (Zhang et al. 2009). This process is required for microtubule extension during growth cone remodeling and axonal growth (Grabham et al. 2007). The nature of many of the proteins affected by DE-71 exposure is suggestive of neuronal damage and plasticity related to repair and homeostatic mechanisms.

PDIA3, also known as ERp57, was chosen for validation studies because of its multiple functions in the processes identified by the proteomics data set. The expression of PDIA3 protein was up-regulated by both 2D DIGE and Western Blot analyses. As a constituent of the endoplasmic reticulum, in conjunction with other chaperone proteins, PDIA3 assists in glycoprotein folding (Bedard et al. 2005). PDIA3 has a role in synaptic plasticity (Hofmann and Kirsch 2012). A common mode of action could be expected between chemically induced

neurotoxicity and neurodegenerative diseases. In this context, it has been reported that PDIA3 acts as a carrier protein for β -amyloid and that plaque formation in Alzheimer disease may be due to faulty ER posttranslational processing (Erickson et al. 2005). Similarly, it has been proposed that PDIA3 may be involved in α -synuclein accumulation in Parkinson's disease (Wilhelmus et al. 2011).

PBDEs belong to the family of organohalogen chemicals that are ubiquitous around the world, persistent in the environment, and of continued concern in health issues. Previously our lab conducted a similar proteomics study on the polychlorinated biphenyl mixture (PCB), Aroclor 1254 (Kodavanti et al. 2011). In studies with these structurally related compounds, we reported an increase in proteins related to energy metabolism, chaperone function, neuronal plasticity and neuronal damage, similar to what was found with the PBDE mixture in this study (highlighted in Figure 4). Disruptions in PKC activation, calcium signaling, oxidative stress, and thyroid hormone perturbations are recognized as adverse outcome pathways for PCB exposure (Kodavanti 2005; Kodavanti et al. 1998; Figure 4). In the present study the MARCKS protein was down-regulated by DE-71. MARCKS is a widely distributed primary substrate for PKC in the cytoplasm and is involved in neurite outgrowth and dendritic spine morphology during development (Calabrese and Halpain 2005). The Aroclor 1254-dependent decrease in PKC could have been due in part to a lack of the MARCKS substrate, suggesting that a similar loss in PKC activity could occur following DE-71 exposure with possible effects on the nervous system development (Figure 4). The adverse effects for these two groups of chemicals included both morphometric and functional changes. With Aroclor 1254 exposure, we have reported that both dendritic growth and branching was decreased following developmental exposure (Lein et al. 2007), along with decreased motor activity, and learning and memory deficits (Eriksson and

Fredriksson 1996). In agreement with effects of Aroclor 1254, suppression of thyroid hormone mediated dendritogenesis and dendritic branching pattern (Ibhazehiebo et al. 2011) along with decreased motor activity and memory deficits have also been reported with PBDE exposure (Herbstman et al. 2010). Thus we propose common adverse-outcome pathways within this extended family of chemicals (Figure 4). Current proteomic data support the idea that neurotoxic effects observed following developmental DE-71 exposure could arise from the disruption of normal ontogenetic pattern of nervous system growth and development through perturbation of intracellular signaling pathways, causing oxidative stress, and disrupting energy homeostasis.

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Figure Legends

Figure 1. Representative 2D DIGE gels from the cerebellum and hippocampus showing proteins whose expression changed following developmental exposure to DE-71. The corresponding spots (differentially expressed proteins are listed in Supplemental Material, Table S1) are shown on the left panels; the right panels are used for illustration purposes displaying Cy-2, Cy-3, or Cy-5 fluorescently labeled protein spots. The arrows point to the proteins that were altered by DE-71 exposure as determined by the DeCyder 2D software (GE Healthcare). Variation in spot colors occurring with individual gel staining is corrected for with the DeCyder 2D software (GE Healthcare). Red hued spots represent proteins with higher expression in the treated sample, while green hued spots are proteins with higher levels in the control sample. Whitish spots represent proteins whose expression level did not change with DE-71 exposure. The sample size is three controls and three treated brains ($n = 3$). Four proteins were altered in the cerebellum versus 70 proteins in the hippocampus following DE-71 exposure.

Figure 2. Three dimensional visualization of protein spots differentially expressed in the cerebellum (A) and hippocampus (B) of DE-71-treated rats, compared to control samples. Examples show how high (panel A left; 2C, ENOA) and low (panel A right; 1C, HNRH2) expression changes or up- (panel B left, 40H, G3P) and down-(panel B right, 6H, MARCKS) regulation were identified by the DeCyder 2D software. Details of the proteins shown in this figure are included in Supplemental Material, Table S1.

Figure 3. Ingenuity™ Pathway Analysis showing functional correlates of hippocampal proteins that were affected following developmental exposure to DE-71. Panels A and B show the most highly correlated network (score 25) and a merged network of the next 3 highest ranked networks (scores of 17, 9 and 2), respectively. Differentially expressed proteins are in shades of red, with color intensity related to level of change. Networked proteins (from the Ingenuity™ database) are shown in white. Associated canonical overlays are depicted in blue.

Figure 4. The possible adverse outcome pathways for the developmental neurotoxicity of PCBs/PBDEs using a system's biology approach. The figure highlights the pathways that link PCB/PBDE exposure to neurochemical changes and genomic and proteomic changes that may

lead to structural and functional changes. Proteins in red color were altered by the developmental exposure to PCB. Although different proteins were altered by these structurally related chemicals (PBDEs and PCBs), it is very interesting to note that similar pathways were altered by the developmental exposure and hence may have a common mode of action for the adverse effects associated with these chemicals. The numbers in the figure indicate references related to those effects. ¹Kodavanti et al., 2005; 2010. ²Current results; Kodavanti et al., 2011. ³Ibhazehiebo et al., 2011. ⁴ Rice et al., 2007; Herbstman et al., 2010; Eskenazi et al., 2013.

Figure 1.

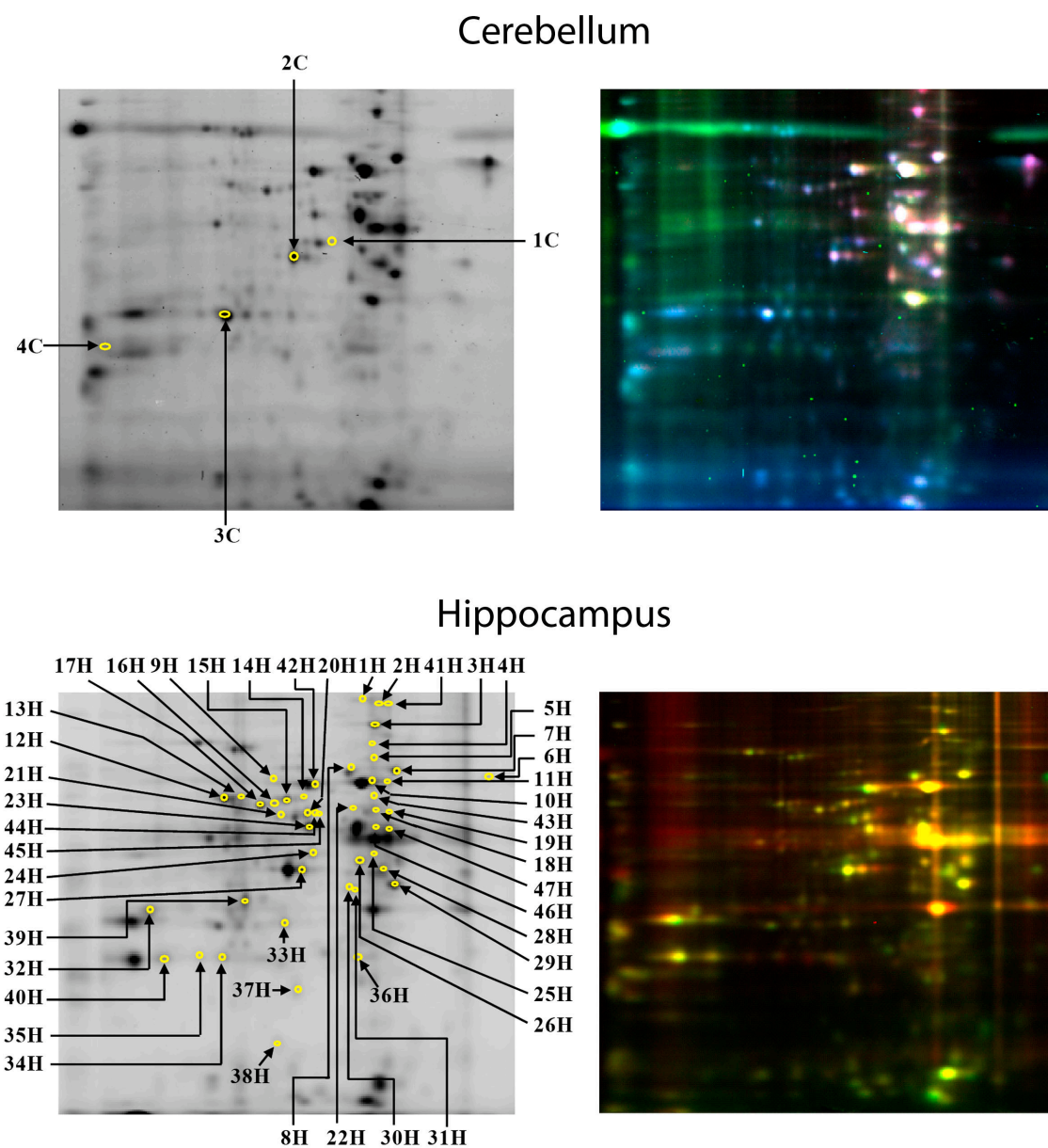
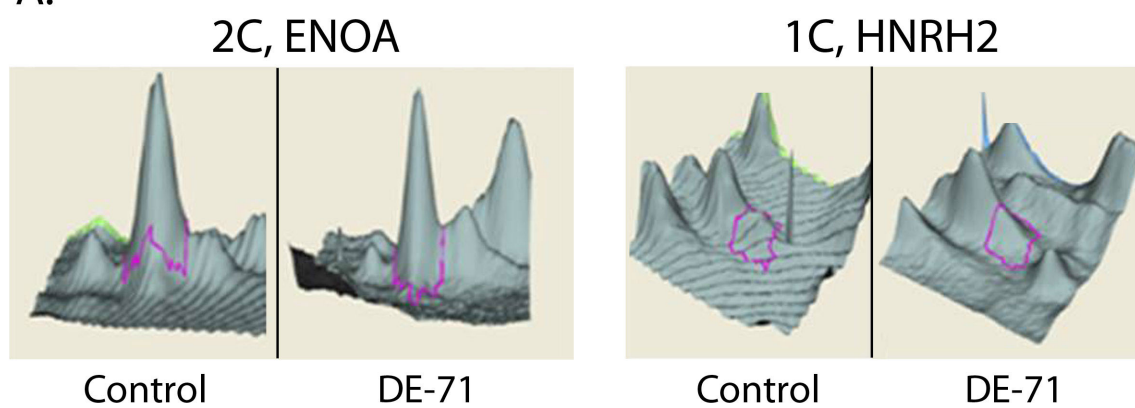


Figure 2.

A.



B.

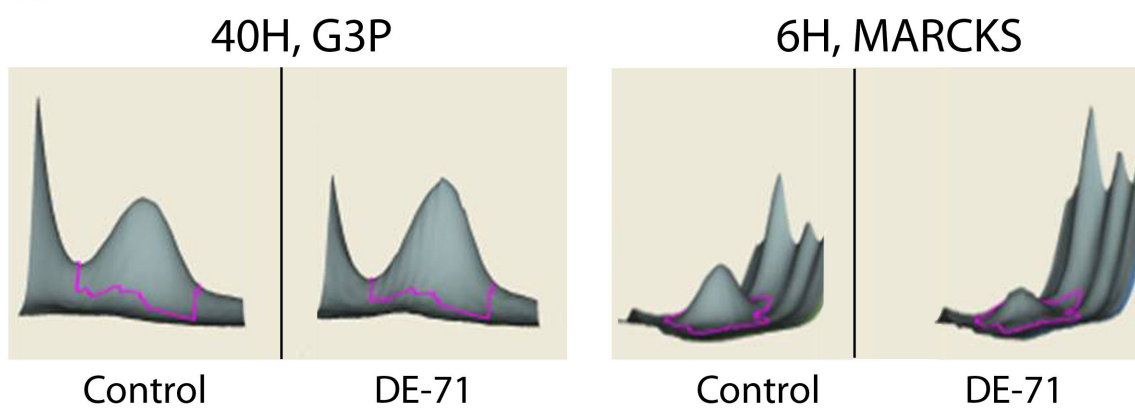


Figure 3.

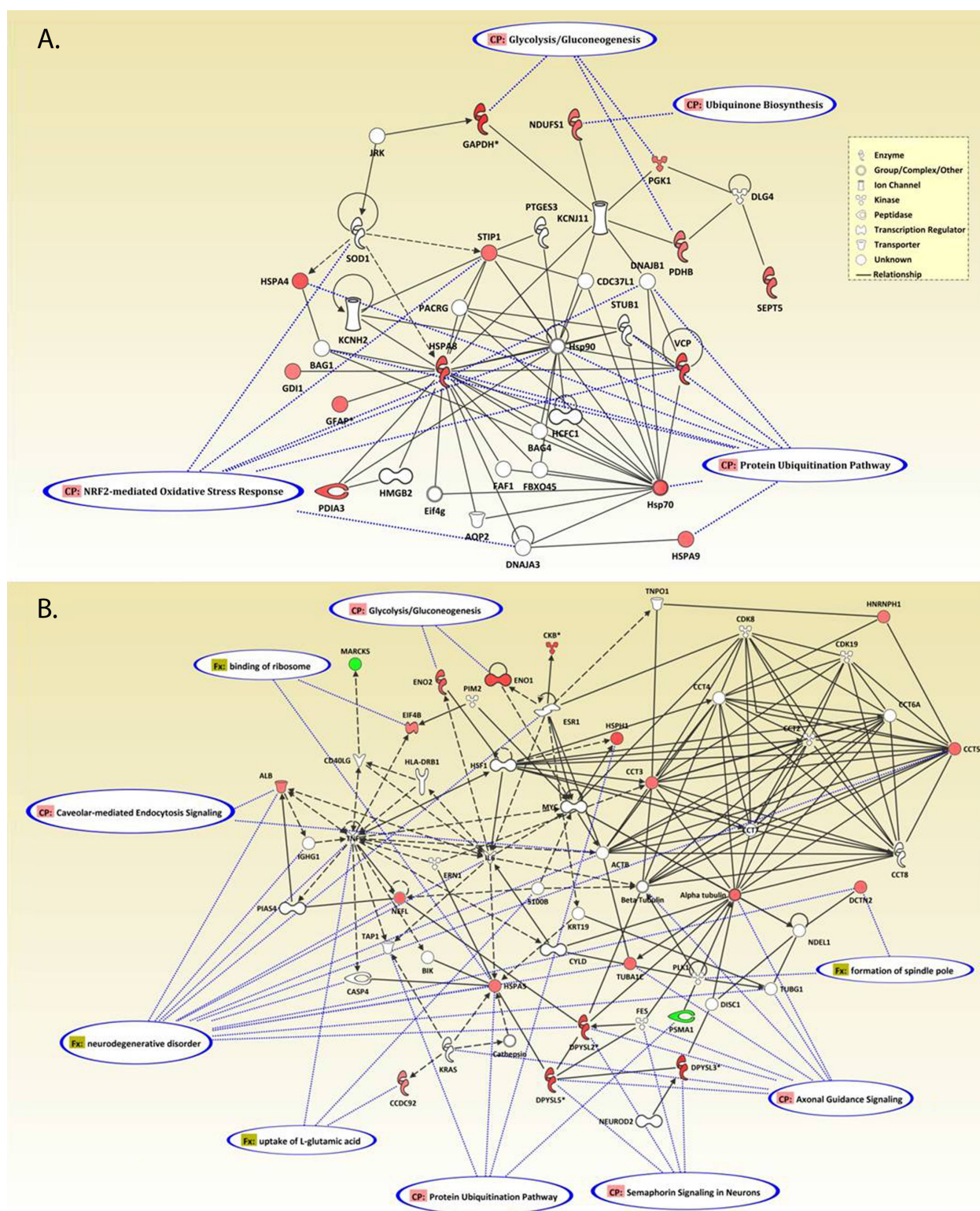


Figure 4.

